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Review article

Clearing or subverting the enemy: Role of autophagy in protozoan infections

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ABSTRACT

The protozoan parasites are evolutionarily divergent, unicellular eukaryotic pathogens representing one of the essential sources of parasitic diseases. These parasites significantly affect the economy and cause public health burdens globally. Protozoan parasites share many cellular features and pathways with their respective host cells. This includes autophagy, a process responsible for self-degradation of the cell's components. There is conservation of the central structural and functional machinery for autophagy in most of the eukaryotic phyla, however, *Plasmodium* and *Toxoplasma* possess a decreased number of recognizable autophagy-related proteins (ATG). *Plasmodium* noticeably lacks clear orthologs of the initiating kinase ATG1/ULK1/2, and both *Plasmodium* and *Toxoplasma* lack proteins involved in the nucleation of autophagosomes. These organisms have essential apicoplast, a plastid-like non-photosynthetic organelle, which is an adaptation that is used in penetrating the host cell. Furthermore, available evidence suggests that *Leishmania*, an intracellular protozoan parasite, induces autophagy in macrophages. The autophagic pathway in *Trypanosoma cruzi* is activated during metacyclogenesis, a process responsible for the infective forms of parasites. Therefore, numerous pathogens have developed strategies to impair the autophagic mechanism in phagocytes. Regulating autophagy is essential to maintain cellular health as adjustments in the autophagy pathway have been linked to the progression of several physiological and pathological conditions in humans. In this review, we report current advances in autophagy in parasites and their host cells, focusing on the ramifications of these studies in the design of potential anti-protozoan therapeutics.

1. Introduction

Protozoa are unicellular organisms that live as parasites or as free-living eukaryotes and interact with some environments and bodies. Every year, protozoal infections cause significant economic and public health burdens globally [1]. Of all the protozoa of medical importance, Apicomplexa make up a considerable group causing several human diseases including leishmaniasis, malaria and toxoplasmosis [1,2]. Protozoan parasites have complex life cycles with completely different host species. The different environments of different hosts and a different response of the same parasite species to a given host require enhanced adaptations and differentiation of parasite.

In recent years, autophagy in protozoan parasites has emerged as critical mechanisms during their biological processes. However, whether autophagy during parasite differentiation can also lead to autophagic cell death, remain elusive [3]. Autophagy is a housekeeping process essential for the maintenance of the metabolic balance in eukaryotes through autophagosomes and lysosomes upon nutrient limitation, infections and other physiological/pathological conditions [4]. Although autophagy is a self-digestion process, it also hastens the degradation of pathogens in a specialized form of autophagy termed xenophagy [5,6]. Xenophagy is the process whereby there is degradation of intracellular microorganism inside a phagosome of cells [7,8] or freely in the cytosol [8]. That is, xenophagy is ordinarily occurs as a result of the presence of a pathogen within the cytosol or vacuole of the host cell. In both cases, a double membrane bound autophagosome engulfs the pathogen [9]. Another type of autophagy known as Light Chain 3 (LC3)-associated phagocytosis (LAP) can also be triggered during intracellular pathogenic infection. This type of autophagy includes the use of LC3 in addition to other parts of the canonical

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autophagy pathway to pathogens that are already contained within a phagosome [9]. Pathogens have autophagic pathway that functions at specific stages of their life-cycles mainly during the differentiation processes. Autophagy also occurs when pathogens are treated with anti-parasitic drugs, as a mechanism to overcome the pressure from the toxic compounds [10–13]. In addition, intracellular parasites have the capacity to manipulate host-cell autophagy in order to control the infection within a host [8].

The prioritization of the responses remains to be understood. There is observed sequestration of pathogens in the host cell autophagic machinery in an attempt to dodge endocytic and phagocytic processes. There can be a fusion between the vesicles in the endolysosomal pathway and the whole pathogen or autophagosome containing pathogen to deliver ligands for adaptive or innate immune activation, or with the lysosome for degradation [14].

For pathogens that supplant autophagy-like pathways, it is likely the cellular components involved are different from the known autophagy and might have evolved some exclusive adaptive mechanisms. Additionally, autophagy inside the pathogen itself is a uniquely defined virulence strategy for eukaryotic microorganisms [14]. In protozoan infections, the role of autophagy has been argued due to conflicting reports. One group has suggested parasites escape host cell defenses using autophagy, while the other supports the claim that the host makes use of autophagy to degrade the pathogens [15,16]. Despite the debate, there is no reservation that the autophagic process resolutely affects the pathogenesis and virulence of protozoan infections. Therefore, this mechanism may offer a promising therapeutic target for drug discovery [17]. Furthermore, the interplay between host autophagy and intracellular pathogens has distinct outcomes depending on the type of microorganism and host cell [8]. Many pathogens can be engulfed and degraded in an autolysosome by xenophagy. Another group of microorganisms can evade the autophagic pathway to its advantage. Some microbes prevent the autophagic flux from increasing in an autophagic niche which does not fuse with lysosomes [18], while, others have evolved to live and replicate inside an autophagic compartment with autolysosomal characteristics [8,19]. The autophagy in protozoan parasites has led to the exploration of the physiological triggers and functions of autophagy during parasite infections [20]. This work revises the involvement of protozoan parasites and hosts cell autophagy during parasite infection.

2. The role of host autophagy machinery in controlling *Plasmodium* infection

The *Plasmodium* parasite is increasingly gaining resistance to almost all the antimalarial drugs and therefore there is an immediate need to identify new therapeutic targets to enable the discovery of unique, effective and safer drugs for the disease treatment [21]. These parasites have several similar cellular features and pathways including autophagy with their host cells. Autophagy functions in both innate and adaptive immunity, including immune activation, infected cell survival, immune cell homeostasis, and pathogens degradation (Fig. 1). In addition to immune signal regulation, autophagy also plays a role in innate and adaptive immune activation [14]. Although autophagy is well studied in red blood cell development, little is known about autophagy in *Plasmodium*-infected erythrocytes [22].

It has been suggested that in *P. falciparum*, all the three forms of cell death namely apoptosis, autophagy and necrosis occur [23]. Autophagy was first observed in *P. falciparum*- and *P. vivax* infected human liver tissues by De Brito et al. in 1969 [24]. They reported the presence of different sizes of vacuoles, bound by single or double membranes, and identified them as malaria pigment [24]. With advances in molecular biology techniques, the role of autophagy in *Plasmodium* is gradually becoming understood [21]. The primary system and mechanism for autophagy are evolutionarily conserved in most of the eukaryotic organisms, however, *Plasmodium* and *Toxoplasma* have a reduced collection of known autophagy-related proteins. Additionally, *Plasmodium* lacks orthologs of the initiating kinase ATG1/ULK1/2, and both lack proteins involved in the nucleation of autophagosomes [22]. Apicomplexan parasites also do not have lysosomes, so they instead breakdown the autophagosome load in vacuoles with a proteolytic function. For example, in *Plasmodium*-infected erythrocytes, autophagosomes fuse with the food vacuole that is responsible for degrading hemoglobin that the parasite imports from the red blood cells cytosol [25]. Sporozoites and merozoites from *Plasmodium* are the invasive stages for hepatocytes and erythrocytes, respectively, but they do lack a food vacuole. However, it has been proposed that after hepatocytes invasion, *P. berghei* micronemes decorated with ATG8 are removed from the parasite and dissipated by enzymes in the parasitophorous vacuole (PV) [22,26]. A better understanding of how autophagy is regulated in *Plasmodium* species has resuscitated due to the recent report of artemisinin-resistance mutations occurring in *P. falciparum* Atg18 (PfAtg18) [27].

Furthermore, the accumulation of vacuoles during chloroquine administration might be due to the inhibition of autophagy by chloroquine [21], which is further linked to the alterations in PfATG8 distribution [28]. Some of the roles of autophagic proteins (ATG) in *Plasmodium* includes, vesicular trafficking, programmed cell death and apicoplast maintenance [29], though the roles of several plasmodial Atg remain obscure. During protein secretion, *Plasmodium* upon entry into a host cell, undergoes full remodeling of the host cell by carrying its proteins into the host cell via parasite plasma membrane and parasitophorous vacuoles (PV) (Fig. 1) [21]. A recent report has shown that PfAtg8 is mainly located at the apicoplast membrane of *Plasmodium* throughout liver- and blood-stages, but without evidence of autophagic membranes surrounding the apicoplast [30]. Therefore, it suggests that Atg8 may not be associated with the autophagic degradation of the apicoplast [21]. Also, if there is a delivery of proteins or lipids by autophagic machinery to the growing apicoplast membrane, component proteins expression is mainly by the trophozoite stage [31]. Evidence for expression and localization of *Plasmodium* Atg from various sources are shown in Table 1.

The level of immunity in the liver, its regenerative ability and increased metabolic activity makes it a perfect environment for the rapid-growing parasite [32]. The 6-cysteine domain protein P36 on the surface of sporozoite is the significant determinants for recognition and elimination of liver cells [33]. In *Plasmodium*-infected liver cells, distinct autophagy pathways are stimulated during parasite development. While canonical autophagy operates as an important nutrient source during *Plasmodium* liver stage development, molecular mechanism related to either xenophagy or LAP represent an intracellular immune response, termed as *Plasmodium*-associated autophagy-related (PAAR) response [32]. Thus, as the discussion has been about the role of host autophagy as an associate or adversary during parasite infection at the liver stage [34], an emanating view showing that *Plasmodium* development in the liver could represent PAAR [9,32,35]. The PV membrane (PVM) prevents interaction between the parasite and the cytoplasm of the hepatocyte by serving as a natural barrier. Although, the PVM is from the plasma membrane of host cell, it is clearly altered by the pathogen, which inserts its proteins to this membrane [36]. It is possible that some of these proteins including UIS3, UIS4 interact with cytosolic defense mechanisms and exploit them [9].

However, host cells have developed complex strategies to recognize and invade pathogens in the vacuole. In addition to the endolysosomal and autophagic pathways being important recycling mechanisms that regulate cell homeostasis, mammalian cells utilize this digestive ability to control and remove intracellular pathogens. Intriguingly, this two-faced role strongly affects the development of *Plasmodium* in the liver [32]. In *P. vivax*-infected human hepatocytes, stimulation of interferon-gamma (IFN-γ) enhances LC3 and lysosome engagement to the PVM [37]. *Plasmodium* parasites targeted for clearance by IFN-γ–LAP mainly fail to evade the intracellular immune response. An uncommon feature

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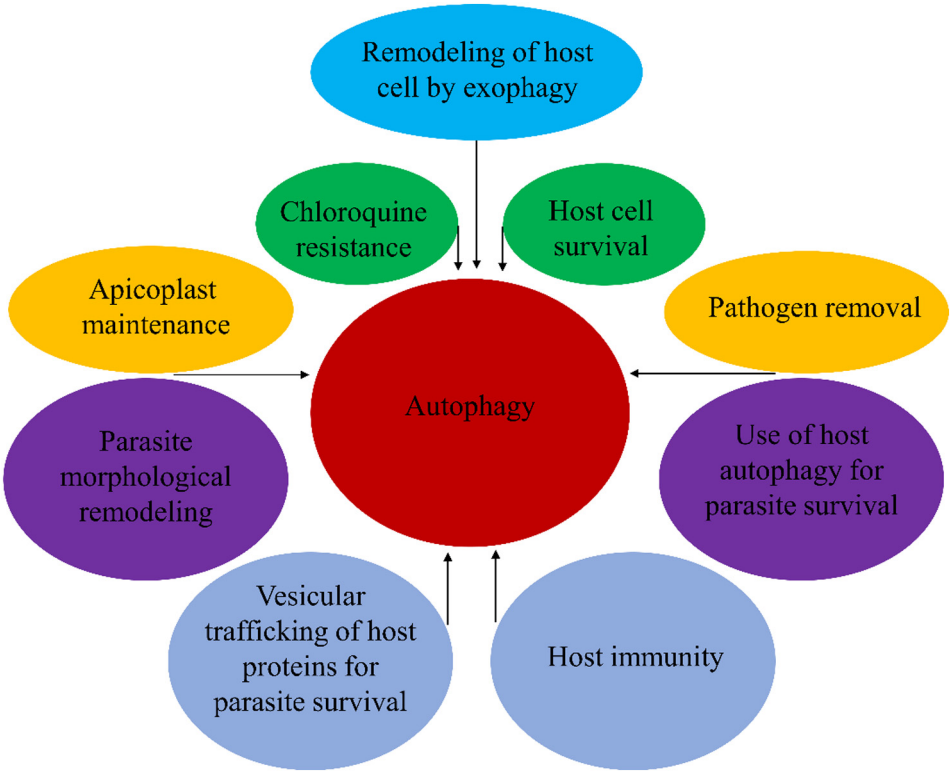

Fig. 1. Autophagy in malaria pathogenesis. Autophagy occurs in both innate and adaptive immunity, including immune activation, infected cells and parasites survival, vesicular trafficking, parasite and host cell remodeling, apicoplast maintenance and pathogens removal.

of *P. vivax* is its capacity to form dormant stages (hypnozoites) in the hepatocytes. It is reasonable to suggest that *P. vivax* prevents an autophagic initiation response by interfering with the signaling pathway of IFN-γ, to facilitate differentiation and persistence of hypnozoite [32]. In *P. berghei*-infected hepatocytes, the PVM is decorated with microtubule-associated protein 1 LC3 (the mammalian orthologue of ATG8), ubiquitin, SQSTM1/p62 and lysosomes in a process resembling selective autophagy [38].

Furthermore, as the development of *P. berghei* is attenuated in host hepatocytes lacking autophagy, it might have led to autophagy occurring at the PVM of the host cell to supply the parasite with necessary nutrients for maximum growth [22,38,39]. In the case of the rodent malaria parasite *P. yoelii*, it has been speculated that the parasite survives in LC3-positive autophagosome-like vacuoles. Therefore, *P. yoelii* prevents the maturation into an autolysosome [40].

Interestingly, it has also been shown that sporozoites can enter liver cells within a transient vacuole, independently of cell traversal (CT), and that pathogens that lack CT within a transient vacuole link up with lysosomes and are cleared [41]. Again, during motility, CT, and invasion, a group of sporozoite factors are secreted, yet the precise role for most secreted factors remains obscure [42]. It will be interesting to know how these sporozoite factors aid the parasite survival in the hepatocyte.

Host cell's canonical autophagy presents an additional nutrient source for the liver stage of *Plasmodium*. The genetic manipulation of macroautophagy pathway of the host cell shows a general decrease in parasite growth [35,38,39]. ATG5-deficient cells are defective in initiating macroautophagy as well as alternative autophagy pathways such as selective autophagy or LAP due to ATG5 being part of the LC3-lipidation pathway [32]. The conditions that lead to LC3 conjugation system upon *Plasmodium* infection and how lipidated LC3 sequester to the PVM remain to be elucidated [9]. However, ATG5 is needed for the recruitment of LC3 to the PVM [35], whiles parasite protein upregulated in infectious sporozoites 3(UIS3) binds to and retains LC3 on the PVM [43]. Furthermore, when LC3 sequester onto the PVM, UIS3 obstruct LC3 binding to other targeted proteins, resulting in autophagy inhibition [43]. Further studies on the LC3-UIS3 interplay and the significance of UIS3 for parasite development is needed towards our understanding of LC3-UIS3 interface. Furthermore, the interaction will serve as a platform for antimalarial drug development. Despite the fact that parasites in ATG5-deficient cells are subject to deprivation of nutrient and thus have retarded development, the composite survival rate of the parasite is enhanced overwhelmingly due to the PAAR response also depending on ATG5 [32,35,38]. Contrastingly, deficiency for ULK-

Table 1
Autophagy proteins during *Plasmodium* life cycle.

Source	Atg1	Atg2	Atg3	Atg4	Atg5	Atg7	Atg8	Atg12	Atg16	Atg18	Atg101	FIP200	Vps15	Vps34
Sporozoite	√	√	√				√		√					√
Ookinete	√	√	√	√	√	√	√		√	√	√	√	√	√
Gametocyte	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Schizont	√	√	√	√	√	√	√	√	√	√			√	√
Trophozoite	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Ring-form	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Merozoite	√						√			√				
Liver-stage			√			√	√							

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associated protein, focal adhesion kinase family interacting protein of 200 kD (FIP200) of host cells lacks only the canonical autophagy pathway. Due to the decreased supply of nutrient, parasite growth is significantly affected [32].

Recent in vivo starvation experiments favor the antagonistic function of the different branches of host cell autophagy on the liver-stage *Plasmodium* development. Starved mice during liver-stage *Plasmodium* infection showed over 20-fold increase in parasite load compared to normal-fed infected mice counterparts. The increased parasite burden can partly be due to additional nutrients resulting from canonical autophagy activation [32]. The significant effect, however, was a drastically increased parasite survival rate upon host starvation [38]. Taken together, recruitment of LC3 to the PVM, parasite protein UIS3 on host autophagy machinery through its non-canonical association with LC3 and the PAAR response could be exploited for therapeutic purposes.

3. *Toxoplasma gondii* manipulates host cell signaling to avoid targeting by autophagy

Upon starvation, the proteins in *Toxoplasma* re-localize from the cytosol to punctate structures that resemble autophagosomes. Prolonged starvation causes significant parasite mortality, as a result of the mitochondrial network disruption in *Toxoplasma* tachyzoites (rapidly dividing forms of the parasite) [22]. Intriguingly, under normal intracellular growth conditions, TgATG8 localizes to the apicoplast membrane [44], similar to that of *Plasmodium*. This unique organelle carries essential metabolic pathways, and cell lines lacking TgATG8 [45] and related proteins TgATG3 and TgATG4 (regulator of TgATG8 membrane association) [22], have similar phenotypes indicating loss of both the apicoplast and parasite viability. Thus, indicating that, part of the autophagy machinery is used for linking TgATG8 to the apicoplast, where it plays an essential function in the inheritance of organelle during cell division [22,45]. This vital role seems different from canonical autophagy and shows that apicomplexan parasites may have partly subverted the machinery for performing a specialized non-canonical task [46]. Interestingly, the residual body of unused material is left behind after *Toxoplasma* tachyzoite invasion following their division by endodyogeny, which disappears during the development of parasites in the vacuole. Thus, the PV might be an essential compartment for the acquisition of nutrient between the parasite and its host cell [22]. *T. gondii* lives in PV within macrophages and prevents their fusion with the lysosome [14]. The interplay between immune-responses resulting in either autophagy from lysosomal vacuole disruption or degradation and the processes used by the pathogen to undermine such responses are major factors that contribute to the outcome of infection [47].

Functional investigation of a *Toxoplasma* ATG9 homolog (a protein potentially crucial for the early stages of autophagosome formation), indicated a possible role for canonical autophagy in the parasitic organisms for stress related survival, either as intracellular parasites or outside host immune cells [48]. Therefore, this evidence indicates that canonical autophagy could be part of an integrated stress response pathway in *Toxoplasma* [22]. Surviving in the host cell, *T. gondii* relies on its capacity to live in a vacuole that avoids lysosomal degradation and allows parasite replication. The interaction between immune-responses that lead to either autophagy resulting from lysosomal vacuole degradation or disruption and the approaches used by the parasite to hide from such responses are major factors to the outcome of infection [47]. The ATG proteins regulate autophagosome formation, which results from the structure of the phagophore, a cytosol-sequestering vesicle with double-membrane that can engulf either portions of the cytoplasm or its content [49]. This is coordinated by upstream kinases including the target of rapamycin (TOR) complex [50], and the Phospoinositol 3-kinase (PI3K)C3. The phagophore then transitions into a fully closed autophagosome that further fuses with lysosomes to form autolysosome, leading to degradation and recycling of the sequestered portions [49].

The IFN-γ is key to increasing the expression of host Guanylate Binding Proteins (GBPs) and the Immunity Related GTPases (IRGs), both required for disrupting pathogen vacuoles by a mechanism yet to be established [9]. Elimination of *T. gondii* in IFN-γ-activated mouse host cells is linked with blebbing, vesiculation and stripping of the PVM early after cell entry [49]. Earlier reports described autophagosome-like double-membrane vacuoles surrounding the parasite in activated macrophages, indicating a function for autophagy in the destruction of the tachyzoites [51]. Also, a dependence on the IRG Irgm3 was reported, which was found to be localized to the autophagosomal membranes covering the naked parasite [9]. TgAtg5 is required for the engagement of Irga6 and Irgb6 to the PV in macrophages, fibroblasts and granulocytes [9]. Lack of TgAtg5 led to Irga6, Irgb6 and Irgd in the host cytoplasm. In connection with TgAtg7 and TgAtg16L1, Irgb6 and mGBPs are recruited to the PV [9]. Similarly, TgAtg3 is necessary for loading of IRGs and mGBP2 onto the PVM and control of Toxoplasma infection [52]. It is possible other GBPs are involved, but, the mechanism is yet to be established. However, the role of TgATGs cannot be underestimated.

Other reports described an accumulation of LC3-positive vesicles closer to the disrupted PV before their elimination in activated host cells. Proteins like ATG9, or ATG14 and Beclin1, that are significant for the autophagosome formation initiation, are dispensable for parasite degradation [52–55]. Therefore, it seems IFN-γ-treated cells can eliminate parasites through a process that is independent of the buildup of canonical autophagosomes. In addition to the recruitment of PV-located LC3, parasites clearance also depends on autophagy proteins which are needed for LC3 lipidation [49]. There is an IFN-γ-independent/CD40-dependent autophagy machinery-related degradation of tachyzoites inside the host cell [56]. Unlike the IFN-γ-dependent killing of *T. gondii*, the CD40-stimulated pathway enhances parasites clearance by the lysosomal system [56]. Furthermore, canonical autophagy appears to be involved, as CD40 signaling seems to act on autophagy (Fig. 2) [49]. CD40 ligation to eliminate *Toxoplasma* needs a synergistic interaction with TNF α, thereby causing a Beclin1 and ULK1 signal to enhance clearance Toxoplasma by autophagy [9,57]. Therefore, exploiting the above phenomenon and further considering the roles TgATGs play will not be far-fetched and that will be key in addressing the therapeutic challenges.

T. gondii tachyzoites infect almost every nucleated cell and survive by living in the PV. Active host cells invasion leads to the production of PV which depends on the parasite actin-myosin motor and successive secretion of proteins from micronemes and rhoptries [47,58,59]. After microneme secretion, *T. gondii* micronemal proteins (MICs) are expressed on the parasite surface and interact with membrane receptors of the host cell [47,60]. Some evidence suggests that *T. gondii* activates host cell signaling that counter-regulates the autophagy machinery to evade clearance [61,62]. The presence of the parasites in a PV leads to a molecular cascade that inhibits *T. gondii* targeting by the autophagy protein LC3 and thus avoiding Beclin1- and Atg7-dependent autophagic clearance via activation of epidermal growth factor receptor (EGFR)-dependent pathways [9,49]. The activation of EGFR and PI3K-regulated Akt pathway, inhibits parasite targeting for autophagic/lysosomal elimination in host cells [61]. Parasite-activated EGFR-dependent signaling also acts on the roles of signal transducer and activator of transcription 3 (STAT3) that can control autophagy through the transcriptional regulation of multiple genes, or can prevent autophagy by sequestering the eukaryotic Initiation Factor 2 alpha (eIF2α) [63]. Following the above reports, another finding suggested that Gefitinib, an EGFR inhibitor, caused significant reduction of parasite replication in HeLa cells [64]. Recent observation further indicates that *T. gondii* infection promotes lipophagy to provide a source of energy for parasite development [65]. Thus, there is the need for further studies to address the interaction between autophagy-*Toxoplasma*. Since autophagy is influenced by different pathways depending on the infected organism,

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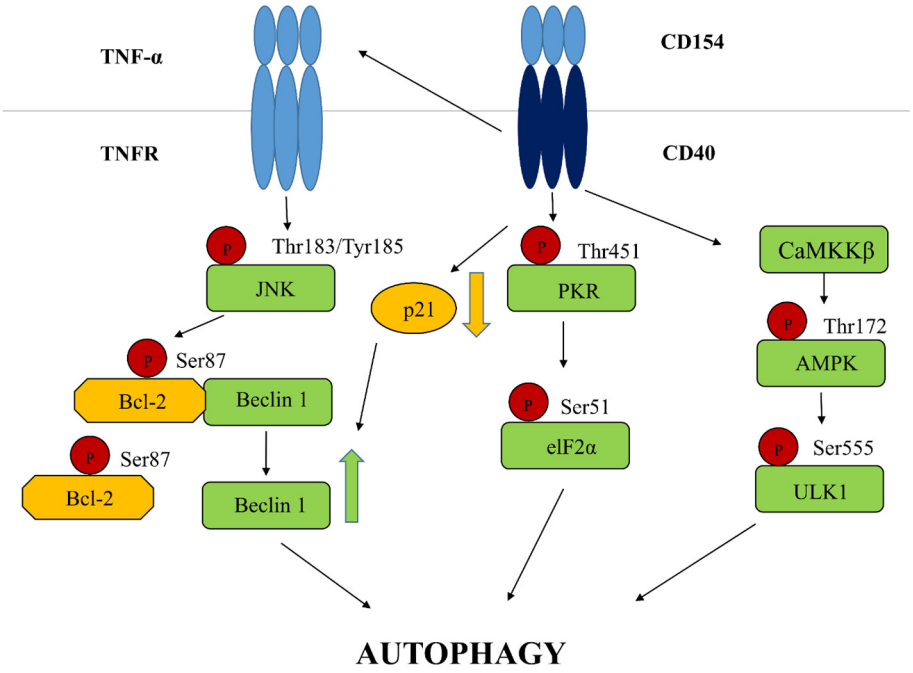

Fig. 2. Autophagy stimulated signaling pathways are activated by CD40. The stimulation is through four mechanisms. CaMKKβ-mediated Threonine-172 AMPK phosphorylation is induced by CD40 which in turn causes Serine-555 ULK1 phosphorylation and ULK1-led autophagy. Activation of PKR and eIF2α by CD40. CD40 may trigger additional mechanisms that act on ULK1 and Beclin 1. *T. gondii* autophagic killing by CD40 that is dependent on ULK1, Beclin 1, PI3KC3, ATG5, ATG7, and lysosomal enzymes. CD40 increases Beclin 1 protein levels possibly via down-regulation of p21. CD40 causes autocrine secretion of TNF-α which in turn causes JNK1/2-led phosphorylation of Bcl-2 at Serine 87 and dissociation of Bcl-2 from Beclin 1. Modified with permission from Subauste [47].

cell type and strain of *Toxoplasma* used for the study, a broad integrated approach will offer appropriate answers/ solutions to the numerous lingering unanswered questions.

4. Evasion and modulation of autophagy by *Leishmania* parasites

Several *Leishmania* species have been implicated in the induction of autophagy [9]. Induction of autophagy is linked with an increase in *Leishmania* parasite load [66] and the acquisition of macromolecules from the host cell [16]. Despite the growing evidence of the autophagic role in exterminating *Leishmania* parasites in macrophages [67], the available evidences are still inadequate to explain the Polymorphonuclear membranes (PMNs) behavior during transmission of the parasite to the host cells [68]. Undoubtedly, the first line of defense against foreign invaders like the pathogens are PMNs, and the survival or failure of infection depends mainly on the proper function of these cells employing several antimicrobial mechanisms like inflammation, oxidative burst, and phagocytosis [68]. The ability to withstand the defense arsenal of the host cell inside the phagolysosome gives the parasites a chance to use the phagocytic cells for transmission and propagation [68]. Earlier studies report how the *Leishmania* parasites use PMNs as the media for transfer to macrophages through the Trojan horse mechanism (act as reservoirs) [68,69].

After the *Leishmania* parasites engulfment, neutrophils can either start eliminating the parasites or act as Trojan Horses for transfer of parasite to the host (act as reservoirs). Therefore, the role of neutrophil during early infection is of great importance as that determines the success or failure of the pathogenesis of the disease [68]. Furthermore, a critical role of neutrophil autophagy mediated by infection or other forms of determining the engulfment of a neutrophil by macrophages has been reported [68]. Some other studies have reported the role of autophagy in infection by the *Leishmania* parasites. For instance, there has been a demonstration that through microautophagy, a large PV acquired macromolecules from the host cell is induced by *L. mexicana* [69].

Furthermore, recent reports indicate that both *L. amazonensis* and *L. major* infections trigger activation of the autophagic pathway in macrophages from susceptible BALB/c mouse [16,67]. Others have shown that in susceptible BALB/c mice macrophages, the autophagic induction led to an increased intracellular load of *L. amazonensis* [70]. There was further indication that starvation-induced autophagy did not change intracellular *L. major* parasitic load in susceptible BALB/c mouse macrophages [70]. Autophagy induction by starvation or cytokines alters parasite load and further evidence suggest the presence of autophagy in the bone marrow of visceral *Leishmania* patient [16]. Database searches have revealed homologs of ATGs in *Leishmania* parasites [3]. Formation of autophagosomes is regulated by well-coordinated action of ATG products. In mammals as well as yeast, autophagosomes are formed by two distinct pathways: one involving ATG8 and the other ATG12 and ATG5. All the ATG proteins of the two different ubiquitination cascades are present in *L. major* [3].

Several studies have shown autophagy of host cell under infection by different *Leishmania* species in an attempt to determine whether it is for defense mechanism or parasite survival [2,67,69,71]. The ability of *Leishmania* parasites to endure, impair and survive the defense machinery of the host cell within the phagolysosome offers opportunities for the parasites to use phagocytic cells for transmission and propagation [68]. The increase of autophagy in human PMNs (hPMNs) after *Leishmania* infection caused Pitale et al. [68] to explore the trigger for autophagy and its effect on the resultant macrophages uptake of the PMNs. Both canonical and noncanonical autophagy were triggered upon infection in which canonical autophagy followed after the non-canonical autophagy [68]. A recent report has demonstrated that knockdown for Atg5 and Atg9 in monocytic cell-line from human, THP-1, reduced *L. donovani* survival, indicating that autophagy is needed to enhance *Leishmania* infection [71].

Interestingly, Atg5 knocked-down in BALB/c macrophages showed enhanced *L. major* parasitic replication [67,69]. Additionally, a new study showed that knockdown for Atg5 in the *L. major*-resistant C57BL/6 macrophages led to an increased parasite load [72], which indicates that an Atg5-dependent autophagic activation process could lead to the clearance of *L. major* intracellular parasites. Also, both *L. amazonensis* and *L. major* have been shown to induce autophagy in CBA macrophages, however, *L. major* infection was indicated to produce a lower percentage of parasite-induced vacuoles decorated by endogenous LC3-II. In *L. amazonensis* and *L. major* autophagy modulation infection in vitro, similar rates were seen in the parasite load or infected cells when autophagy was inhibited or induced [69].

Furthermore, increased levels of LC3-II were linked to autophagy induction, since lower levels of LC3-II were seen in infected cells

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compared to those treated with chloroquine, an inhibitor of autophagic flux [69]. Mitroulis et al. also reported a greater LC3-I to LC3-II conversion in macrophages of bone marrow from a male patient infected with *L. donovani* in comparison to bone marrow from a healthy patient [73]. It has been suggested that the lack of observed LC3-II positivity increased in cells infected with *L. donovani* previously treated with rapamycin might be due to parasite inhibition of classical autophagy (or apoptosis) activation through the PI3K-Akt-mTOR (mammalian target of rapamycin) pathway, but inducing this process through another pathway different from mTOR [71,74–76]. Thus, triggering the autophagic process by the PI3K-Akt-mTOR pathway seems to enhance *Leishmania* spp. intracellular survival, especially in species that survive in a compartment having degradative characteristics. Therefore, induction of autophagy after infection would be harmful to the host, since it would enhance intracellular *Leishmania* viability [69]. Indeed more work is required to elucidate the type of mechanism at play between *Leishmania* species and the host autophagy machinery such that the host is considerably overwhelmed by the *Leishmania*-authophagy interplay.

5. Autophagy in host cells infected with *Trypanosoma* parasites

Various genome database searches together with advanced research reveal the presence of autophagy-associated components in *Trypanosoma cruzi* [8,77]. In *T. brucei*, the main autophagic events are conserved, from the formation of autophagosome to the degradation in lysosomes [20,78]. Again, there is confirmation of autophagy-related functions of several ATG homologs or protein complex: ATG3, ATG5, ATG7, ATG24 and PI3K [78–80]. The *T. cruzi* has two TcAtg8 homologs: TcAtg8.1 and TcAtg8.2 [8]. Using TbATG8.2 as a marker, the formation of autophagosome can be in vitro monitored during chemical treatments and starvation. In cells infected with *T. cruzi*, there is a gradual increase in LC3-positive vesicle number [81]. Induction of LC3-positive tubules in *T. cruzi* infected cells could favor the in or out of specific molecules into the TcPV, creating an enabling environment for the parasite [8]. Intriguingly, autophagy induction requires acidocalcisomes found in trypanosomes and some other protozoan parasites [82]. The induction signaling and degradation functions seem to be governed separately by two organelles in *T. brucei*, induction signaling through the acidocalcisomes and degradation through the lysosomes [20].

The availability of an autophagic-like process in *T. cruzi* was first shown by morphological studies, indicating the presence of double-membrane vesicles and multivesicular structures in trypanocidal treated parasites [8,83]. Further genome database searches together with advanced research analyses indicated the existence of autophagy-associated components in *T. cruzi* as indicated earlier [77,84,85]. *T. cruzi* has all parts of the LC3/Atg8 conjugation system, but not Atg5-Atg12 protein complex, indicating a different manner to initiate and elongate the autophagosome. A recent report showed that TcVps34 kinase is controlled by TcVps15 and that both form a complex that take part in starvation-induced autophagy in *T. cruzi* [86]. Bafilomycin is an H^+ pump inhibitor that prevents the average autophagic flux, by producing numerous autophagic structures. However, in *T. cruzi* bafilomycin impairs the first steps of autophagy, indicated by the low number of Atg8.1 positive vesicles produced under this treatment [8].

Starvation of *T. cruzi* epimastigotes occurs in the gut of the insect vector naturally, thus, autophagy could represent a crucial survival mechanism of the parasite in the gut of the insect vector [3]. Recent report indicates an intense expression of ATG8.1 in differentiating epimastigotes but not normal epimastigotes or in fully developed metacyclic trypomastigotes, suggesting that these cells are undergoing a very dynamic autophagic process [3]. The *T. cruzi* PV is reported to be decorated with LC3 protein and that the autophagic inhibitors wortmannin, 3-methyladenine or vinblastine impairs this recruitment and significantly decreases the intracellular infection. Fascinatingly, the infection is significantly reduced in the cells lacking specific autophagy genes Beclin1 or Atg5, which are needed for initiation of autophagy. This is an indication that autophagic-derived compartments are required for the enhanced penetration of *T. cruzi* into the host cell [8]. Evidence of *T. cruzi* invasion mechanism shows that trypomastigotes use the lysosome-dependent membrane repair strategy to enter the host cell [87]. The rupture of the *T. cruzi* cell surface during invasion may cause the autophagic response as a way to restore the plasma membrane integrity [8]. Thus, autophagy represents an important survival mechanism for *T. cruzi* in both vector and mammalian host.

T. brucei typically resides in the bloodstream of the mammalian hosts or the midgut of the tsetse, both considered as host-environments rich in nutrient. The bloodstream-form of *T. brucei* produces ATP mainly through glucose glycolysis, which is abundant in the blood of the vertebrate hosts. In the procyclic form (which lives in the midgut) where the parasite mitochondrion is fully developed, in addition to glycolysis the ATP can be generated by oxidative phosphorylation [20]. These critical features of *T. brucei* metabolism, show that autophagy in *T. brucei* relies on intracellular ATP level. While autophagy in *T. brucei* can be robustly triggered by amino acid starvation, low energy charge in the cell cannot cause autophagy and an AMPK-mediated energy sensing mechanism is also not involved [88]. See Table 2 for an overview of the different autophagic proteins of the various parasites and host.

Similar to other protozoan parasites, *T. brucei* has to adapt to entirely distinct environments in different hosts. Also, trypanosomes have the mechanism to specifically breakdown glycosomes and probably mitochondria in a process called “glycophagy”. These suggest a role of autophagy in the differentiation and environmental adaptation of African trypanosomes [3,8]. Autophagy-like characteristic, cytoplasmic vacuoles decorated with high concentrations of the ubiquitin-like ATG8.1, can be observed under conditions of serum deprivation.

6. Conclusion

As one of the main processes that control cell survival or death, autophagy has a significant role in the protozoan parasite life-cycle. Evidence indicates that autophagy is needed by parasites to move from one stage to the other. These changes offer the parasite the ability to adapt to the host in their life-cycle, making parasite autophagy an excellent target for therapeutic drugs. Host autophagy has also been shown to regulate parasitic invasion with the tendency effects on the maturation of the PV. All these suggestions indicate that a proper understanding of autophagic interplay between host and protozoan parasites, may lead to finding novel and newer targets for new therapies for protozoan infections. These targets may include LC3 sequestration onto the PVM and obstruction by UIS3 towards binding to other targeted proteins.

List of abbreviations

ATG	autophagy-related protein
CT	cell traversal
EGFR	epidermal growth factor receptor
eIF2α	eukaryotic Initiation Factor 2 alpha
FIP200	focal adhesion kinase family interacting protein of 200 kD
GBP	Guanylate Binding Protein
hPMN	human PMN
IFN-γ	interferon-gamma
IRG	Immunity Related GTPase
LAP	LC3-associated phagocytosis
LC3	Light Chain 3
MIC	micronemal protein
mTOR	mammalian TOR
PAAR	*Plasmodium*-associated autophagy-related
Pf	*Plasmodium falciparum*
PV	parasitophorous vacuole

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Table 2
Different autophagic proteins of host and parasites.

Autophagic protein	*Host*	*Plasmodium*	*Toxoplasma*	*Leishmania*	*Trypanosoma*
Atg1 (ULK1)					
Atg2					
Atg3					
Atg4					
Atg5					
Atg7					
Atg8					
Atg9					
Atg10					
Atg11					
Atg12					
Atg13					
Atg14					
Atg16					
Atg18					
Atg23					
Atg24					
Atg101					
Beclin 1(Atg6)					
FIP200					
Vps15					
Vps34					

Protozoan parasites ULK1/ATG1 do not have the activity domain and most likely cannot initiate autophagy induction via the ULK1/ATG1 pathway.

PI3K Phospoinositol 3-kinase
PMN Polymorphornuclear membrane
PVM PV membrane
STAT3 signal transducer and activator of transcription 3
Tc *Trypanosoma cruzi*
Tg *Toxoplasma gondii*
TOR target of rapamycin
UIS3 upregulated in infectious sporozoites 3

Consent for publication

Not applicable.

Authors' contributions

GGK developed the idea. GGK, BA, FAN, YKO and EKA wrote and read the manuscript. All authors approved the final version of the manuscript.

Declaration of competing interest

Authors declare no competing interest.

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